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Extracellular vesicles for drug delivery☆

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ABSTRACT

Extracellular vesicles (EVs) are cell-derived membrane vesicles, and represent an endogenous mechanism for intercellular communication. Since the discovery that EVs are capable of functionally transferring biological information, the potential use of EVs as drug delivery vehicles has gained considerable scientific interest. EVs may have multiple advantages over currently available drug delivery vehicles, such as their ability to overcome natural barriers, their intrinsic cell targeting properties, and stability in the circulation. However, therapeutic applications of EVs as drug delivery systems have been limited due to a lack of methods for scalable EV isolation and efficient drug loading. Furthermore, in order to achieve targeted drug delivery, their intrinsic cell targeting properties should be tuned through EV engineering. Here, we review and discuss recent progress and remaining challenges in the development of EVs as drug delivery vehicles.

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1. Introduction

Extracellular vesicles (EVs) are nano-sized membrane vesicles, released by many, if not all, cell types. EV release has been found to occur in many unicellular- as well as multicellular organisms, suggesting that it represents an evolutionary-conserved process. Mammalian

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cells can release distinct types of EVs, including exosomes, microvesicles, and apoptotic bodies. This classification is based on their intracellular origin. Exosomes are thought to be the smallest vesicle type, with sizes ranging from 40 to 100 nm. They originate from intraluminal budding of multivesicular endosomes (MVE) and are released upon fusion of MVEs with the plasma membrane. In contrast, microvesicles (also referred to as ectosomes) are more heterogeneous in size and can be larger (50 nm-1 μm), and are formed through direct budding from the plasma membrane. When cells are compelled to undergo apoptosis, a heterogeneous population of vesicles (50 nm-5 μm) is released, which are named apoptotic bodies [1]. Despite these differences in origin, no uniform EV nomenclature exists as of yet, due to the overlap in vesicle sizes and the absence of subtype-specific markers. As a result, it remains difficult (if not impossible) to purify and thereby distinguish between vesicle types [2]. In this review, we therefore refer to all vesicle subtypes as extracellular vesicles (EVs).

The cargo of EVs comprises small and long, coding and non-coding RNAs (e.g. mRNA, miRNA, lncRNA), lipids and proteins [3,4]. Initially, EVs were thought to act as 'garbage bags', with a main function in discard of cellular waste. Over the last decade, however, scientific interest in EVs has rapidly increased, after it was shown that biological information packaged in EVs could be transferred between cells, and alter the recipient cells' phenotype. Cells can package a distinct set of biomolecules into EVs via endogenous sorting mechanisms, and release EVs constitutively or after stimulation [5,6]. EVs may subsequently be internalized by target recipient cells, resulting in transfer of mRNAs and miRNAs, which can result in production or silencing of target proteins, respectively [7–9], and of proteins, including membrane proteins [10,11]. EVs can be isolated from bodily fluids, including blood, urine, cerebrospinal fluid and saliva [12–14]. As their content reflects the status of the donor cell, EVs may be applied in diagnostics, either as pathological biomarkers or to follow treatment efficacy (reviewed in [15,16]). In addition, it has become clear that, through their important role in intercellular communication, EVs affect various processes involved in health and disease. The discovery that EVs make up a natural mechanism for information transfer between cells has stimulated interest into their potential use as a new drug delivery platform.

Despite considerable research in the last 50 years, the clinical translation of conventional drug delivery platforms has been limited. The efficiency of these platforms to overcome barriers in macromolecule drug transport, such as reaching the target tissue and engaging intracellular targets, is still unsatisfactory [17]. In addition, concerns related to immunogenicity and toxicity of non-natural delivery systems remain. EVs on the other hand seem to have many features of an ideal carrier system. The EV cargo is naturally protected from degradation in the circulation [18]. EVs seem to possess intrinsic cell targeting properties, and are able to overcome natural barriers such as the blood-brain barrier [19,20]. Furthermore, it is likely that EVs utilize endogenous mechanisms for uptake, intracellular trafficking and subsequent delivery of their content in recipient cells [21]. Importantly, EVs may be nearly non-immunogenic when used autologously. Moreover, several clinical trials using EVs for immunotherapy have already demonstrated the safety of EV administration in humans [22–24].

Although EVs hold immense promise for therapeutic drug delivery, clinical applications may critically depend on the development of scalable EV isolation techniques and approaches for efficient drug loading. Furthermore, improved methods to modify their *in vivo* biodistribution, which is an important determinant of their therapeutic effect as it enables more specific drug delivery to target tissues, are required. In this review, we discuss new findings and recent improvements on these issues, and summarize recent successes in the use of EVs as drug delivery vehicles in animal disease models.

2. Isolation of extracellular vesicles

One of the prerequisites for clinical application of EVs is standardization of the isolation process with regards to yield, reproducibility, and purity [25]. Furthermore, such an isolation method should be scalable and robust. For large scale EV production, the manufacturing process sequentially involves expansion of the donor cell line, collection of the conditioned medium, and EV purification. Thus far, several methods for the isolation of EVs have been described. The most commonly used method is differential ultracentrifugation (UC). EVs are isolated based on sedimentation at high *g*-forces. Generally, this method comprises low speed spins to remove cell debris, followed by high speed spins to pellet EVs. Sucrose density gradients may subsequently be utilized to separate vesicle types based on density, and to further purify vesicles from protein aggregates [26,27]. Disadvantages of UC and sucrose gradients include the time-consuming protocol, operator-dependent yield, and possible aggregation and rupture of EVs due to high shear forces [28]. Isolation of EVs using UC may therefore not be useful for clinical practice, and novel isolation techniques are topic of intensive investigation. Two distinct approaches for isolation can be discriminated.

The first approach, immunoaffinity isolation, is based on selective capture of EVs that bear specific marker proteins on their surface. This could be important when separation of EV subtypes is required, although it is currently unknown whether specific subtypes are more or less feasible for drug delivery purposes. Clayton et al. developed an isolation method to capture EVs derived from antigen-presenting cells (APCs) using antibody-coated magnetic beads. Using antibodies specific for major histocompatibility complex (MHC) class II, a specific EV subtype (i.e. exosomes) could be isolated [29]. A different antibody-based method to isolate EVs was described by Ashcroft et al., who used a microfluidic circuit to isolate CD41-positive platelet-derived EVs in plasma. EVs were captured with an anti-CD41 antibody-coated mica surface [30]. This standardized and quick method requires a very low amount of plasma (10 μl) and could be adjusted for other sources of EVs in the future. However, the absence of well-defined EV markers may thus lead to isolation of only specific EV subsets or EVs derived from specific cell types, and successful elution of intact EVs from the beads might prove challenging. Furthermore, immunoaffinity isolation protocols are not very attractive for clinical applications, since EVs are isolated at a very small scale.

The second approach comprises methods that isolate EVs based on their size. With these methods, considerable efforts have already been made to improve scalability of EV isolation. Lamparski et al. showed increased recovery of MHC class II-expressing EVs using a combination of ultrafiltration and ultracentrifugation of EVs in a 30% sucrose/deuterium oxide cushion, for the first time showing that it is possible to isolate EVs for clinical application [31]. However, a drawback of this method is the low EV recovery, hindering application in large clinical studies. In an attempt to isolate EVs using filtration techniques only, Heinemann et al. developed an easy three-step protocol [32]. First, a 0.1 μm pore size polyethersulfone (PES) membrane was used to remove dead cells and cell debris. The sample was then passed through a 500 kDa molecular weight cut-off modified PES filter to remove free proteins and reduce large volumes, followed by isolation of EVs using a 0.1 um Track Etch filter. A comparison of sequential filtration versus UC showed that although filtration resulted in a slight reduction of EV yield compared to UC, it resulted in isolation of a more specific subset of EVs. The major advantage of this method is the fast and fully automatable protocol, although non-specific EV protein binding to the membranes leading to lower recovery may present a limitation.

The use of size-exclusion chromatography (SEC) for EV isolation from plasma was first described by Boing et al. [33]. Fractionation of plasma using a sepharose CL-2B column resulted in fast and specific separation of proteins, HDL, and EVs. SEC isolation also resulted in a higher recovery of EVs compared to UC. Increased standardization of EV isolation was reported by Welton et al., who employed a